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Effects of nalmefene hydrochloride on TLR4 signaling pathway in rats with lung ischemia-reperfusion injury

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Abstract. – OBJECTIVE: To investigate the effect of nalmefene hydrochloride on TLR4 signaling pathway in rats with lung ischemia-reperfusion injury.

MATERIALS AND METHODS: Altogether 64 pure inbred male SD rats were divided into groups A, B, C, and D according to the principle of body weight similarity, with 24 rats in each group. Four groups of rats were respectively twisted on the left testis to establish unilateral testicular torsion rats. Group A was the control group, treated with normal saline, group B was the nalmefene hydrochloride high-dose group, treated with 20 μg/kg of nalmefene hydrochloride, group C was the nalmefene hydrochloride low-dose group, treated with 10 μg/kg of nalmefene hydrochloride, and group D was the sham operation group. Lung tissue was collected 60 h later. Western blotting was used to detect the expression levels of HMGB1, TLR4, CD14, and NF-κB protein, qPCR was used to detect the mRNA expression level, and enzyme-linked immunosorbent assay (ELISA) was used to detect the expression levels of inflammatory factors IL-17, IL-6, and ICAM-1.

RESULTS: The expression levels of HMGB1, TLR4, CD14, NF-κB protein, mRNA, IL-17, IL-6, and ICAM-1 in group A were significantly higher than those in groups B, C, and D ($p<0.05$), while were significantly lower in group D than in groups B and C ($p<0.05$), and were significantly lower in group B than in group C ($p<0.05$).

CONCLUSIONS: Nalmefene hydrochloride can effectively inhibit the signal pathway of TLR4, and can effectively reduce the injury caused by lung ischemia-reperfusion. The large dose is closely related to the good effect, which is worthy of promotion.

Key Words:
Lung ischemia-reperfusion, Nalmefene hydrochloride, TLR4, CD14, NF-κB, Signal pathway.

Introduction

Ischemia-reperfusion is a phenomenon that often occurs during major operations and organ transplantation. During this period, in the reperfusion process, the tissue of specific organs will aggravate the tissue damage to specific organs during reoxygenation, which is considered to be more harmful than ischemia itself[1]. Ischemia-reperfusion injury of lung is related to lung transplantation, extracorporeal circulation, pulmonary embolism extirpation, and pneumonectomy, which may lead to pulmonary dysfunction and serious damage. Among its pathogenesis, oxygen free radicals, inflammatory mediators, and neutrophils play an important role[2]. As the mechanisms involved in lung ischemia-reperfusion injury are complex and interrelated, showing one of the clear mechanisms can help prevent potential serious complications[3]. Toll-like receptor (TLR), a pattern recognition receptor that provides the first line of defense against pathogens such as bacteria and viruses, is the initial site for activation of inflammatory signals in the lung during ischemia and reperfusion[4]. TLR4 plays a key role in the inflammatory cascade of lung ischemia-reperfusion injury. The downstream molecular nuclear factor-κB (NF-κB) of the cascade is also important for autoimmune regulation. The changes of various proinflammatory factors, chemokines, adhesion molecules, and enzymes involved in lung ischemia-reperfusion injury depend on the activation of NF-κB[5-7]. Activation of TLR4 leads to nuclear translocation of NF-κB, which in turn leads to an increase in the expression level of various inflammatory factors, resulting in injury[8,9]. High mobility group protein B1 (HMGB1) participates

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in many inflammatory reactions in the body and can combine with TLR4 to promote the activation of NF-κB, which is an important pathway leading to the activation of inflammatory[10]. However, the receptor CD14 of lipopolysaccharide can cause activation of TLR4, thus causing inflammatory reaction[11].

Drug therapy has always been a preferred method to regulate the signal pathway of TLR4 and reduce the injury caused by lung ischemia-reperfusion. However, studies have found that opioid peptides can interact with receptors on the surface of pulmonary capillary endothelial cells and play an important role in acute lung injury[12]. However, some studies have shown that nalmefene hydrochloride, a new generation opioid receptor antagonist, has longer acting time, stronger membrane permeability, higher bioavailability, and less adverse reactions, and can effectively reduce lung ischemia-reperfusion injury[13], so it is commonly used in lung injury reperfusion. However, this research focused on mechanism analysis, and analyzed the influence of nalmefene hydrochloride on TLR4 signal pathway in lung ischemia-reperfusion injury through the rat model.

Materials and Methods

Selection of Experimental Animals

Sixty-four healthy male SD rats were selected, with SPF grade and similar age, weight, and body length. The rats were from Hunan SJA Laboratory Animal Co., Ltd. The animal manufacturing license was SCXK (Hunan) 2019-0003. The laboratory feeding environment was as follows: 12 h of light, 45%-60% of relative humidity, and 23-25°C. This study was approved by the Ethics Committee.

Establishment of Animal Model

Sixty-four rats were randomly divided into groups A, B, C, and D with 16 rats in each group by random number table method. Group A was the control group. The left porta of lung was dissociated after thoracotomy, and the occlusion was removed after 45 min of clamping. The swelling of lung tissue was observed after 5 min. Color recovery showed that the lung ischemia-reperfusion model was successfully established. Reperfusion was continued for 2 h. Blood was collected through the right common carotid artery. Blood samples were collected and then the rats were executed, the upper lobe tissue of the left lung was collected for examination[14]. Group B was a large dose group of nalmefene hydrochloride. Nalmefene hydrochloride (trade name: Jinmeifen, Yuxi Pharmaceutical Co., Ltd., Lingbao, Henan province, China, specification: 1 ML: 0.1 mg, approval number: Chinese medicine standard H20080805) was injected intravenously 10 min before the left pulmonary hilum was blocked. The treatment in other places was the same as that in Group A. Group C was a small dose group of nalmefene hydrochloride. A total of 20 μg/kg of namefene hydrochloride (10 μg/kg) was injected intravenously into the tail before the left lung was blocked for 10 min. The treatment in other places was the same as that in Group A[15]. Group D was a sham operation group. The left hilar was dissociated by thoracotomy without clamping. At the same time, the tail vein of each group was injected with equal volume of 0.9% sodium chloride solution. After 60 h, rats were executed, and lung tissue was extracted.

Detecting of Protein Level by Western Blotting

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) of the same specimen was taken as an internal reference, the animals of each experimental group (n=6) were executed 24 h after modeling, and the lung tissues of the extracted mice were placed in a refrigerator at –80°C for later use. Western blotting was used to detect the expressions of HMGB1, TLR4, CD14, and NF-κB proteins. HMGB1/GAPDH, TLR4/GAPDH, and NF-κB/GAPDH represent the relative expression level.

Detection of MRNA Expression of TLR4, NF-κB, HMGB1, CD14 by QPCR

Extracting of total RNA

About 50 mg of tissue was put into 1.5 ml of RNAse-free centrifuge tube, 0.5 ml of TRIzol was added. After being ground to homogenate by a homogenizer, 0.5 ml of TRIzol was added for placing, and the whole process was about 0.5 h. A total of 200 μl chloroform was added to every 1 ml TRIzol. After rapid shaking and mixing for 30 s, the mixture was placed on ice for 5 min. Then, it was centrifuged at 4°C for 15 min at 12000 r/min. About 400-600 μl of supernatant was transferred to a new centrifuge tube with a pipette gun, then 500 μl/1 ml of TRIzol isopropyl alcohol was added, covered, reversed repeatedly, mixed evenly, placed for 10 min, put into a centrifuge, and

centrifuged at 4°C for 10 min at 12000 r/min. The supernatant was discarded, isopropyl alcohol was absorbed. Then, 1 ml of 75% ethanol was added, and the mixture was thoroughly mixed. The RNA was washed after centrifuging at 4°C for 5 min at 10000 r/min. The supernatant was discarded, dried naturally for 5-10 min, and 20 μl of DEPC water was added to fully dissolve the total RNA.

qPCR

After total RNA was extracted by TRIzol method, cDNA was synthesized by reverse transcription. 2 μg of RNA, 4 μL of RTbuffer(5×), 1 μL of 10 mmol dNTPs, 1 μL of N9 random primer, 1 μL of reverse transcriptase, 1 μL of RNA enzyme inhibitor, 2 μL of 0.1 mol/ L DTT, and 20 μL of DEPC treated water were added to the reaction system, respectively. After mixing, the reaction was carried out at the following temperatures: 37°C for 1 h; cDNA was obtained at 95°C for 5 min. QPCR (quantitative polymerase chain reaction) amplification was then performed. Primers were designed by Shanghai Sangon Biotech (Shanghai, China). PCR amplification reaction conditions: pre-denaturation at 95°C for 10 min; 40 amplification cycles (95°C, 10 s; 6°C, 20 s; 72°C, 25 s); dissolution (72°C-95°C, 0.5°C, 10 s/time). The upstream and downstream primers are shown in Table I. After that, the ratio of the four groups to the internal reference β-actin was compared to obtain the mRNA levels of the four groups.

Detection of Inflammatory Factors IL-17, IL-6, ICAM-1 by ELISA

About 50 mg of tissue was put into 1.5 ml of RNAse-free centrifuge tube, 0.5 ml of normal saline was added, then the homogenate was fully ground by a homogenizer, 0.5 ml of normal saline was added, and then the supernatant was centrifuged at 12000 r/min, and the supernatant was taken for 5 min for enzyme-linked immunosorbent assay (ELISA) analysis. The tests were carried out in strict accordance with the operating instructions of IL-17 ELISA test kit (Wuhan Herostart Biotechnology Co., Ltd., Wuhan, Hubei, China), IL-6 ELISA test kit (Shanghai Tongwei Industry Co., Ltd., Shanghai, China), and ICAM-1 test kit (Shanghai Kalang Biotechnology Co., Ltd., Shanghai, China). About 100 L of standard solution, sample to be tested, and negative and positive control solution were absorbed into the reaction well. A total of 100 L of biological reaction antibody solution was added, covered with film, mixed, and placed for 40 min. Then, 100 μl of streptavidin was added to each reaction well, covered with a film, mixed evenly, and placed for 40 min. The liquid in the reaction well was discarded, washing liquid was added to each reaction well, the mixer was slowly shaken for 1 min, the liquid in the reaction well was discarded, and the steps were repeated for 5 times. A 100 L of reaction substrate A reaction solution and B reaction solution were added to each reaction well, covered with a film, and placed in darkness for 5 min. A 100 μl of termination solution was added into the reaction well, and finally enzyme-labeled analyzer was used to detect the OD (optical density) value of each well at 450 nm wavelength, and the levels of IL-17, IL-6 and ICAM-1 were calculated.

Statistical Analysis

Data analysis was completed by SPSS 18.0 (SPSS Inc., Chicago, IL, USA). The measurement data were expressed in X±S. Single factor analysis was used for the comparison of index differences among groups, and LSD test was carried out. The difference is statistically significant with $p<0.05$.

Results

Basic Data of Four Groups of Rats

There was no significant difference among groups A, group B, group C, and group D in basic conditions, such as weight, age, length, and left testis taken from the four groups of rats ($p>0.05$; Table II).

Table I. Upstream and downstream primers for TLR4, NF-κB, HMGB1, CD14.

Factor	Upstream primer	Downstream primer
TLR4	5′-CCGAGGGCCCACTAAAGG-3′	5′-GCTGTTGAAGTCACAGGAGACAA-3′
NF-κB	5′-AGA-GAAGCACAGATACCACTAAG-3′	5′-CAGCCTCATAGAAGCCATCC-3′
HMGB1	5′-ATGGGCAAAGGAGATCCTA-3′	5′-ATTCTCATCATCTCTTCT-3′
CD14	5′-AAATGGCTTGCTCAGGGTAACT-3′	5′-GTAACTTTTGGTCACACTCTCAACA-3′
β-actin	5′-ATACGCTGGGATGAGCACTGG-3′	5′-TCTTTGCGGATGTCCACGTC-3′

Table II. Some Basic data of four groups of rats (X±S) (n = 16).

Group	Group A	Group B	Group C	Group D	F	*p*-value
Weight (g)	168.23±11.24	165.94±14.06	167.23±12.78	166.22±13.46	0.156	0.926
Age (d)	37.04±2.02	36.84±2.33	38.04±1.99	37.54±2.41	1.440	0.236
Length (cm)	17.46±1.22	17.52±1.42	17.06±1.72	17.36±1.52	0.457	0.713
Indoor temperature (°C)	23.34±1.02	23.78±0.97	23.03±1.34	23.14±1.01		
Indoor relative humidity (%)	49.23±15.32	48.83±16.42	50.23±14.45	49.23±15.32		

Expression Levels of HMGB1, TLR4, CD14, and NF-κB Proteins

The expression levels of HMGB1 protein in groups A, B, C, and D were (0.55± 0.01), (0.43± 0.02), (0.23± 0.01), (0.11± 0.01), respectively. The level of group A was significantly higher than that of groups B, C, and D ($p<0.05$), while that of group D was significantly lower than that of groups B and C ($p<0.05$), and that of group C was significantly lower than that of group B ($p<0.05$). The TLR4 levels of groups A, B, C, and D were (0.31± 0.02), (0.14± 0.01), (0.19± 0.02), (0.04± 0.01), respectively. The level of group A was significantly higher than that of groups B, C, and D ($p<0.05$), while that of group D was significantly lower than that of groups B and C ($p<0.05$), and that of group C was significantly lower than that of group B ($p<0.05$). CD14 levels in groups A, B, C, and D were (1.23± 0.02), (0.51± 0.02), (0.97± 0.01), (0.33± 0.02), respectively. The level of group A was significantly higher than that of groups B, C, and D ($p<0.05$), while that of group D was significantly lower than that of groups B and C ($p<0.05$), and that of group C was significantly lower than that of group B ($p<0.05$). The expression levels of NF-κB protein in groups A, B, C, and D were (1.35± 0.20), (0.59± 0.08), (0.84± 0.13), (0.31± 0.04), respectively. The level of group A was significantly higher than that of groups B, C, and D ($p<0.05$), while that of group D was significantly lower than that of groups B and C ($p<0.05$), and that of group C was significantly lower than that of group B ($p<0.05$; Table III).

MRNA Expression Levels of HMGB1, TLR4, CD14, and NF-κB in Four Groups

mRNA expression level of HMGB1 in four groups

The mRNA expression levels of HMGB1 in groups A, B, C, and D were (1.65± 0.11), (0.59± 0.02), (0.41± 0.03), (1.11± 0.04), respectively. The mRNA expression level of HMGB1 in group A was significantly lower than that in groups B, C, and D ($p<0.05$), while that in group D was significantly higher than that in groups B and C ($p<0.05$), and that in group C was significantly lower than that in group B ($p<0.05$; Figure 1).

mRNA expression levels of TLR4 in four groups

The mRNA expression levels of TLR4 in groups A, B, C, and D were (9.65± 0.21), (3.41± 0.13), (7.24± 0.22), (1.11± 0.14), respectively. The mRNA expression level of TLR4 in group A was significantly higher than that in groups B, C, and D ($p<0.05$), while that in group D was significantly lower than that in groups B and C ($p<0.05$), and that in group C was significantly lower than that in group B ($p<0.05$; Figure 2).

mRNA expression level of CD14 in four groups

The mRNA levels of CD14 in groups A, B, C, and D were (1.62± 0.36), (1.07± 0.13), (1.27± 0.20), (0.37± 0.03), respectively. The level of IL-1β in group A was much higher than that in groups B, C, and D ($p<0.05$), while that in group D was much lower than that in groups B and C ($p<0.05$). There was no difference between group B and group C ($p>0.05$; Figure 3).

Table III. Expression levels of HMGB1, TLR4, CD14, and NF-κB protein in four groups (X±S) (n = 16).

Group	Group A	Group B	Group C	Group D	F	*p*-value
HMGB1	0.55±0.01	0.23±0.01	0.43±0.02	0.11±0.01	3560.00	<0.0001
TLR4	0.31±0.02	0.14±0.01	0.19±0.02	0.04±0.01	369.10	<0.0001
NF-κB	1.23±0.02	0.51±0.02	0.97±0.01	0.33±0.02	8409.00	<0.0001
CD14	1.35±0.20	0.59±0.08	0.84±0.13	0.31±0.04	192.40	<0.0001

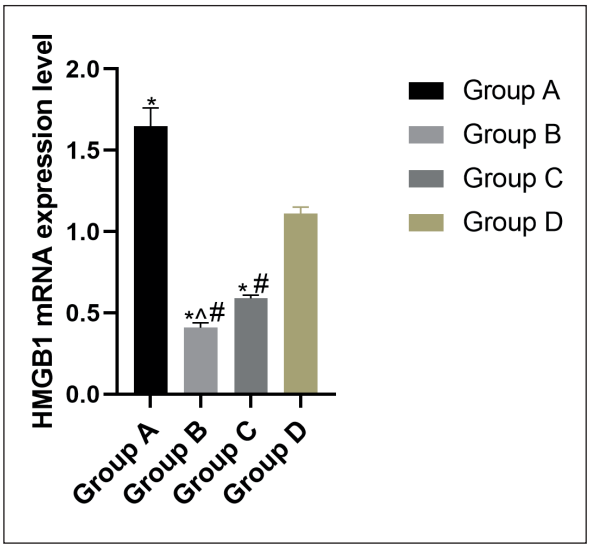

Figure 1. Comparison of mRNA expression levels among four groups of HMGB1. The mRNA expression level was detected by qPCR. The mRNA expression level of HMGB1 in group A was significantly lower than that of group B, C, and D (p<0.05), while that of group D was significantly higher than that of group B and C (p<0.05), and that of group C was significantly lower than that of group B (p<0.05). Note: * indicates that compared with group D, p<0.05; # indicates that compared with group A, p< 0.05; ^ indicates that compared with group C, p<0.05.

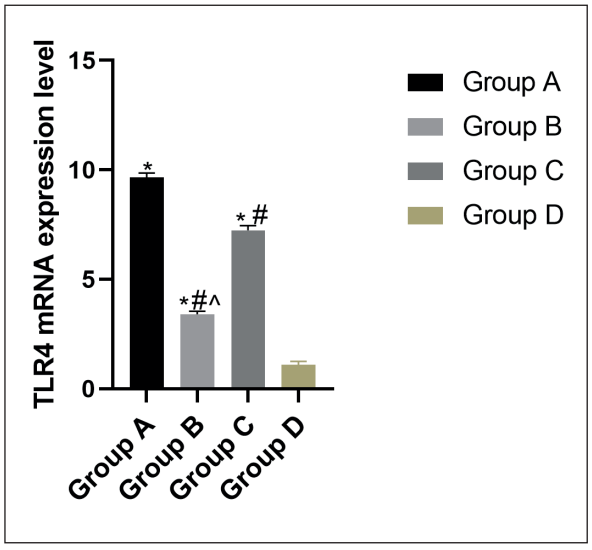

Figure 2. Comparison of MDA content in four groups. MDA content was detected by Bradford. The MDA content in group A was significantly higher than that in group B, C, and D (p<0.05), while the MDA content in group D was significantly lower than that in group B and C (p<0.05). There was no difference between group B and group C (p>0.05). Note: * indicates that compared with group D, p<0.05; # indicates that compared with group A, p< 0.05; ^ indicates that compared with group C, p<0.05.

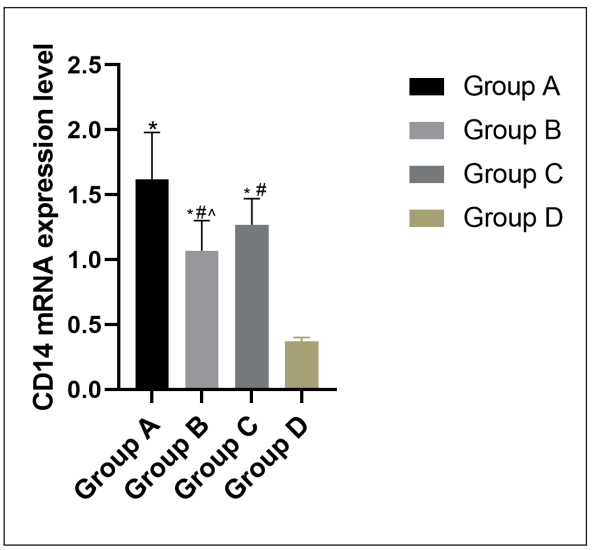

Figure 3. Comparison of NIHSS scores of two groups of patients. hs-CRP expression level was analyzed by ELISA. The level of IL-1β in group A was much higher than that in group B, C, and D (p<0.05), while that in group D was much lower than that in group B and C (p<0.05). There was no difference between group B and group C (p>0.05). Note: *indicates that compared with group D, p<0.05; # indicates that compared with group A, p< 0.05; ^ indicates that compared with group C, p<0.05.

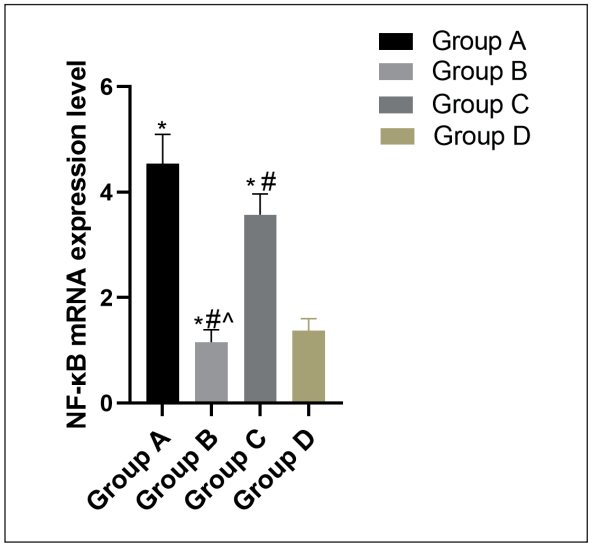

Figure 4. Comparison of the expression levels of inflammatory factor hs-CRP in four groups before and after treatment. The expression level of hs-CRP was analyzed by ELISA. The hs-CRP level in group A was much higher than that in group B, C, and D (p<0.05), while the activity in group D was much lower than that in group B and C (p<0.05). There was no difference between group B and group C (p>0.05). Note: * indicates that compared with group D, p<0.05; # indicates that compared with group A, p< 0.05; ^ indicates that compared with group C, p<0.05.

mRNA expression level of NF-κB in four groups

The mRNA levels of NF-κB in groups A, B, C, and D were (4.54± 0.56), (1.16± 0.23), (3.57± 0.40), (1.37± 0.23), respectively. The mRNA level of NF-κB in group A was much higher than that in groups B, C and D (p<0.05), while the activity in group D was much lower than that in groups B and C (p<0.05). There was no difference between group B and group C (p>0.05; Figure 4).

Levels of Inflammatory Factors IL-17, IL-6, and ICAM-1 in Four Groups

The levels of IL-17 in groups A, B, C, and D were (7.76± 1.06) μg/L, (6.47± 0.29) μg/L, (6.37± 0.22) μg/L, (5.74± 0.15) μg/L, respectively. The level of group A was significantly higher than that of groups B, C and D (p<0.05), while the level of group D was significantly lower than that of groups B and C (p<0.05). The level of group B was significantly lower than that of group C (p<0.05). The levels of IL-6 in groups A, B, C, and D were (10.73± 2.10) μg/L, (5.25± 1.31) μg/L, (7.29± 1.26) μg/L, (2.94± 1.17) μg/L, respectively. The level of group A was significantly higher than that of groups B, C and D (p<0.05), while the level of group D was significantly lower than that of groups B and C (p<0.05). The level of group B was significantly lower than that of group C (p<0.05). The ICAM-1 levels in groups A, B, C, and D were (448.31±22.01) ng/mL, (237.66±24.05) ng/mL, (358.76±22.95) ng/mL, and (167.76±25.05) ng/mL respectively. The level of group A was significantly higher than that of groups B, C, and D (p<0.05), while the level of group D was significantly lower than that of groups B and C (p<0.05). The level of group B was significantly lower than that of group C (p<0.05; Table IV).

Discussion

Lung ischemia-reperfusion injury occurs mostly after surgery, which is of great harm and complicated pathology. Patients often show pulmonary edema, increased pulmonary vascular resistance, pulmonary hypertension, and other symptoms, and even die in severe cases[16,17]. This investigation aimed to study the effect of nalmefene hydrochloride on TLR4 signal pathway.

Studies have found that opioid drugs have inhibitory effects on the transmission of TLR4 signal pathway. Opioid nalmefene can block the signaling pathway of TLR4, effectively remove oxygen free radicals and various pathogenic factors in the body, and improve ischemia-reperfusion[18-20]. TLR4 can provide a new target for clinical treatment of the pathogenesis of lung injury. The more serious the lung ischemia-reperfusion injury, the higher the levels of TLR4 and proteins on its signaling pathway, such as HMGB1, CD14, NF-κB, and mRNAs, as well as the expression levels of various inflammatory factors[21-23]. In this study, we tested the protein expression level and mRNA expression level of TLR4, HMGB1, NF-κB, and CD14, and found that the protein expression level and mRNA expression level of TLR4, HMGB1, NF-κB, and CD14 in BC groups were significantly lower than those in group A, and the expression level in group B was significantly lower than that in group C. In a study on intracranial injury, Li et al[24] also found that nalmefene can reduce serum factors such as IL6. We hypothesized that it was the inhibition of TLR4 and its pathway proteins by nalmefene that led to a decrease in the level of corresponding inflammatory factors. So, in this research, the expression levels of inflammatory cytokines IL-17, IL-6, and ICAM-1 were also detected after ischemia-reperfusion. It was found that the levels of IL-17, IL-6, and ICAM-1 in group A were significantly higher than those in groups B, C, and D, while the levels in group D were significantly lower than those in groups B and C. The level of group B was significantly lower than that of group C. According to the relevant data of group A and group D, in pulmonary ischemia-reperfusion of rats, the more severe the symptoms of pulmonary ischemia-reperfusion, the higher the levels of TLR4 and proteins on

Table IV. IL-17, IL-6, ICAM-1 level of group A, B, C, D (X±S) (n = 16).

Group	A	B	C	D	F	*p*-value
IL-17 (μg/L)	7.75±1.07*	2.47±1.06*#^	5.37±0.92*#	1.74±0.55	143.20	<0.0001
IL-6 (μg/L)	10.73±2.10*	5.25±1.31*#^	7.29±1.26*#	2.94±1.17	76.91	<0.0001
ICAM-1 (ng/mL)	448.31±22.01*	237.66±24.05*#^	358.76±22.95*#	167.76±25.05	450.20	<0.0001

Note: * indicates that compared with group D, p-value <0.05; # indicates that compared with group A,p-value < 0.05; ^ indicates that compared with group C, p-value <0.05.

its pathway, and the higher levels of IL-17, IL-6, ICAM-1, and other cytokines. From the data of group B, group C and their comparison with group A, it can be seen that nalmefene hydrochloride can alleviate the lung ischemia-reperfusion injury by inhibiting the expression levels of TLR4 in lung ischemia-reperfusion of rats, thus significantly reducing the expression levels of related inflammatory factors. From the comparison of data between group B and group C, the higher the dose of the drug, the more significant the inhibition of TLR4 pathway, the better the relief effect on pulmonary ischemia-reperfusion injury, and the more the level of inflammatory factors decreased. Wang et al[25] in the rat model, found that nalmefene alleviated ischemia-reperfusion injury and reduced inflammatory factors in serum, which was similar to the results of this study.

This research discussed the influence of nalmefene hydrochloride on some signal pathways and cytokines, and suggested the therapeutic effect of nalmefene hydrochloride on lung ischemia-reperfusion injury according to the influence of these signal pathways and cytokines. However, this speculation did not involve more specific molecular mechanisms such as differential expression of miRNA. Therefore, whether nalmefene hydrochloride causes differential expression of certain miRNA in rats can be discussed in future investigations, and related biological researches can be designed according to this differential expression.

Conclusions

To sum up, nalmefene hydrochloride inhibits the signal pathway of TLR4, reduces the injury caused by lung ischemia-reperfusion and the large dose is closely related to the good effect.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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